Appendix A

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A novel HBV DNA vaccine based on T cell epitopes and its potential therapeutic effect in HBV transgenic mice

Xiangming Li[1], Xiaofeng Yang[1,2], Yunyun Jiang[1] and **Jing Liu[1]**

[1]Department of Molecular and Cell Biology, School of Life Sciences, University of Science and Technology of China, Hefei, Anhui 230027, People's Republic of China
[2]Department of Stomatology, Medical University of South Carolina, 173 Ashley Avenue, BSB 441,Charleston, SC 29407, USA

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Abstract

DNA vaccination represents a novel therapeutic strategy for chronic hepatitis B virus (HBV) infection. Recently, some HBV DNA vaccines have been used in the preliminary clinical trials and exhibited exciting results in chronic HBV carriers. But these vaccines only encoded the single viral antigen, the S or the PreS2/S antigen. In this study, we designed a polytope DNA vaccine encoding multiple T cell epitopes. We found that it induced stronger CTL responses than the vaccine encoding the single antigen in H-2^d and H-2^b mice, although the CTL response to L^d-restricted epitope suppressed the CTLs to other epitopes in H-2^d-restricted mice. Interestingly, heat shock protein 70 as an adjuvant not only enhanced CTL response to the viral antigen but also overcame this epitope suppression. Furthermore, the polytope DNA vaccine resulted in a long-term down-regulation of hepatitis B virus surface antigen and inhibition of HBV DNA replication in a HBV transgenic mouse model. Therefore, our research indicates that it is practicable and feasible to design a polytope DNA vaccine for chronic hepatitis B immunotherapy.

Introduction

Despite the exisltence of the therapeutic drugs and the widespread use of the subunit recombinant vaccines for prophylaxis, hepatitis B virus (HBV) has been a serious threat to human health until now, particularly in developing countries, with an estimated 370 million chronic carriers (1, 2). Persistent chronic HBV infection may result in cirrhosis and hepatocellular carcinoma, with high mortality rates (1). Hepatitis B patients whose infection has been eradicated develop an effective long-lasting polyclonal, multispecific immune response. This response is not seen in patients with persistent HBV infection, and these patients also show the diminished CD4$^+$ T_h responses to hepatitis B virus surface antigen (HBsAg) and weak or undetectable CD8$^+$ CTL responses to multiple hepatitis B epitopes (3, 4). Therefore, the principal goal for the people chronically infected with HBV should be to stimulate a successful immune response, which will result in a long-term viral clearance. These observations have led to the concept of the therapeutic vaccination stimulating specific T cell responses (4, 5).

Although the clinical trial results were controversial and preliminary, HBV subunit recombinant protein vaccines were proved to be effective in some chronic HBV infection patients (6–8). In a clinical trial, vaccination elicited PBMC proliferative responses specific for envelope antigen in 7 of 27 chronic HBV carriers, and these responses were mediated by CD4$^+$ T lymphocytes (6). Compared with recombinant vaccine, DNA vaccine is a better technique to stimulate specific cellular immune responses and is effective in the mouse strains that respond poorly to protein subunit vaccines (5, 9), so it may represent an alternative therapeutic approach for chronic HBV infection. In the phase I clinical trial, HBV PreS2/S DNA vaccine primed antigen-specific T cell responses in healthy people (10). Recently, DNA vaccination with PreS2/S was used for chronic HBV infection therapy in the preliminary clinical trial and showed the exciting results that it induced vigorous T cell responses and eliminated the persistent infection (11).

A classical DNA vaccination plasmid encodes a single antigen; however, this may not offer the ideal strategy for CTL activation because it does not contain the sufficient numbers of CTL epitopes. Epitope-based vaccination, compared with the single-antigen immunization, spans the HLA diversity of a target population and primes immune responses against the

Correspondence to: J. Liu; E-mail: leexm@mail.ustc.edu.cn

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multiple epitopes; moreover, it avoids epitope drift in the case of viral infections and even triggers the required type of immune response (12). Therefore, the polytope DNA vaccine technique was developed (13). In this approach, multiple alphabeta $CD8^+$ CTL epitopes derived from several antigens were conjoined into single artificial constructs in a 'string-of-beads' or linear fashion (13–15). Recently, the polytope DNA vaccination has been shown to induce efficient cellular immunity in different models of viruses and tumors (16–18). Moreover, it primed much stronger CTL responses than those based on the single protein antigen (19). Therefore, the polytope DNA vaccine may have an important advantage over the single-antigen-based DNA vaccines for HBV therapy.

Heat shock proteins (hsp) had once been considered as 'chaperokines' (20) since they have both chaperon and cytokine abilities (21). No matter how its ability to directly activate dentritic cells was probably due to the contaminant of endotoxin (22, 23), it was confirmed that hsp70 molecule was able to mediate antigen-specific CTL responses by a $CD4^+$ T cell-independent pathway (24–26). In the previous study, hsp73 was proved to enhance CTLs to each epitope in multispecific $CD8^+$ T cell responses by delivering them to processing pathways for MHC class I-restricted presentation in H-2^d mice (27). Thus, hsp is an attractive innate adjuvant in vaccine formulations to enhance its immunogenicity for CTLs.

In this study, we designed a DNA vaccine based on multiple CTL and T_h epitopes for therapeutic immunization and examined its immunogenicity in H-2^d and H-2^b MHC-restricted mice models. To improve antigen-presenting and specific CTL responses, hsp70 molecule was fused to polytope antigen as a genetic adjuvant. Finally, we evaluated its potential therapeutic effects in a HBV transgenic mouse model.

Methods

Vector construction

pCI vector (Promega, Madison, WI, USA) was used to construct DNA vaccine plasmids. An artificial polytope antigen comprised continuous one universal T_h Pan DR epitope [(PADRE) sequence] and four HBsAg CTL epitopes (S_{28-39}, $S_{198-209}$, $S_{172-191}$ and $S_{208-216}$). Each CTL epitope was followed by AAA nucleotides encoding lysine. IgG κ chain leader sequence was used as signal peptide, and inserted Kozak sequence at the 5′-terminal of signal peptide as the ribosome-binding site, so it generated the polytope vaccine plasmid, pMulE.

Six overlapping oligos, averaging 60–65 nucleotides in length of 15 nucleotide overlaps, were synthesized (Takara, Dalian, China). The final polytope antigen gene was spliced together using splicing by overlap extension and PCR techniques. Dimers were made of synthetic oligonucleotides 1 and 2, 3 and 4 and 5 and 6 (0.4 pg of each) in 40-μl reactions containing standard 1× *Pfu* PCR buffer, 0.2 mM deoxyribonucleotide triphosphates and 1 U of *Pfu* DNA polymerase (hot start at 94°C) using the thermal program: 94°C for 10 s, 42°C for 20 s and 72°C for 20 s for five cycles. After five cycles, the PCR program was paused at 72°C and 20-μl aliquots of reactions 2 and 3 were mixed (reaction 1 was left in the PCR machine) and subjected to another five cycles. At cycle 10, the program was paused again and 20 μl reaction 1 was added to 20 μl reaction from the combined reactions 2 and 3, and another five cycles were completed. Two 20-mer oligonucleotides were used to amplify the gel-purified full-length product using the above reaction mixed at an annealing temperature of 50°C for 25 cycles. The full-length gel-purified PCR fragment was cloned into pCI vector to make the polytope vaccine plasmid, pMulE.

HBsAg gene was cloned into pCI vector and generated the pS plasmid. Mycobacterial hsp70 gene was a gift from Qian Huang (Whitehead Institute for Biomedical Research, Cambridge, MA, USA). hsp70 gene was cloned into pCI vector and generated the pH70 plasmid. For enhancing CTL activity, hsp70 gene was fused to the C-terminal of polytope antigen gene, inserted into the pCI vector, and then generated the fusion plasmid, pMulE/hsp. All the constructs were sequenced to confirm the introduction of the desired change.

Cell line

P815 and EL4 cell lines were obtained from the Chinese Tissue Culture Collection (Shanghai, China). After being transfected with 1 μg plasmid encoding eukaryotically expressed HBsAg (pCDNA-S) with Lipofectamine (Invitrogen, Carlsbad, CA, USA), P815 and EL4 cells were maintained under continuous selective pressure. Therefore, the cloned P815/S and EL4/S cell lines that displayed stable expression of HBV S protein were generated and used as target cells in standard (6-h) ^{51}Cr release assays.

Mice

Female BALB/c (H-2^d), C57BL/6 (H-2^b) and HBV transgenic mice were kept under the pathogen-free condition in the facility of the University of Science and Technology of China Life Science School. BALB/c and C57BL/6 mice were obtained from National Rodent Laboratory Animal Resources (Shanghai, China). HBV transgenic BALB/c mice, expressing HBV full gene (adr serotype) in mice liver, were purchased from Infectious Disease Center of no. 458 Hospital (Guangzhou, China). All the mice used for experiments were at the age of 8 weeks.

DNA immunization

Vaccine plasmids were purified from the transformed *Escherichia coli* strain DH5αby Wizard PureFection Plasmid DNA Purification System (Promega) and stored at −70°C. Groups of mice ($n = 6$) were inoculated into each tibialis muscle with 50 μl of 10 μM cardiotoxin (Latoxan, Valence, France) and then after 5 days with 100 μg of plasmid DNA in 100 μl 0.9% NaCl saline. After 1 week, all the mice were boosted with the same immunization methods.

CTL assay

The splenocytes from each vaccinated mouse were harvested after lysing red cells with ACK solution (8.3% NH_4Cl/0.17 mol l^{-1} Tris, pH 7.4). The spleen cells were suspended in complete DMEM with 10% FCS. For checking CTL to each epitope, the splenocytes were cultured for 6 days *in vitro* with recombinant murine IL-2 at the final concentration of 10 IU ml^{-1} and re-stimulated by the addition of 1 μg ml^{-1} of the indicated

synthetic peptide (Sangon, Shanghai, China) or the irradiated syngeneic HBsAg-expressing transfectants (10^6 irradiated stimulator cell for 3×10^7 splenocytes). These effectors were used in standard (6-h) ^{51}Cr release assays against the peptide-sensitized target cells (P815 for BALB/c and EL4 for C57BL/6). Target cells were sensitized for 1 h with 10 μg ml^{-1} peptide at 37°C followed by two washes. For checking CTL to HBsAg, the splenocytes were re-stimulated by recombinant IL-2 at the final concentration of 10 IU ml^{-1} and the irradiated syngeneic HBsAg-expressing transfectants. After 5 days, CTLs were harvested and washed. Specific cytolytic reactivity was determined by standard (6-h) ^{51}Cr release assays against syngeneic HBsAg-expressing target cell, P815/S or EL4/S. In these two assays, serial dilutions of the effector cells were cultured with 2×10^3 ^{51}Cr-labeled targets in 200-μl round-bottom wells in triplicate.

T cell proliferation assay

The spleen cells were harvested after lysing red cells with ACK solution and were cultured in triplicate using 96-well round-bottom plates at 5×10^6 cell ml^{-1} in 200 μl RPMI 1640 medium containing 5% FCS, and were re-stimulated with 10 μg ml^{-1} PADRE peptide. After the 72-h incubation, [^{3}H]thymidine ([^{3}H]TdR) was added (1 μCi per well). The cells were incubated for an additional 16 h, and the [^{3}H]TdR incorporation into DNA was measured. The results were corrected for the background activity (Δ counts per minute).

Cytokines secretion assay

The splenocytes were prepared and cultured with PADRE peptide in triplicate by the same operation in the proliferation assays. The culture supernatants were collected on the third day. The concentrations of IFN-γ and IL-2 were measured by ELISA using the commercial cytokine assay kit (Diaclone, Besoncon, France); the limit of this detection was 5 pg ml^{-1}.

Serology assay

The mice were bled after the first injection from tails at different time points. The blood samples were centrifuged at $2700 \times g$ for 20 min and the sera were stored at −70°C for antigen assay. HBsAg in the sera were measured using HBsAg ELISA kit (SABC, Shanghai, China). The sera (diluted at 1 : 10) were added into ELISA plates pre-coated with anti-HBsAg mAb. The plates were incubated at 37°C for 2 h. After washing with phosphate-buffered saline with 0.5% Tween-20 (TPBS) three times, 100 μl peroxidase-conjugated secondary antibody was added to wells and incubated at 37°C for 1 h. After washing with TPBS three times, color was generated by adding *o*-phenylenediamine dihydrochloride substrates, 100 μl 1 M H_2SO_4 was added and the absorbance at 492 nm was measured on an ELISA reader.

Real-time quantitative PCR assay

The HBV transgenic mice were bled after the first injection and 50 μl sera samples was prepared. HBV DNA was checked by HBV DNA PCR-FIUOTEC kit (SABC). The fluorescent signals were examined by PRISM 7000 Quantitative PCR (ABI, Foster city, CA, USA). HBV DNA was calculated by PRISM 7000 SDS software.

Assay for serum alanine aminotransferase activity

Alanine aminotransferase (ALT) was determined by serum transaminase assay kit (Catachem, Bridgeport, CT, USA). After the first injection, HBV transgenic mice were bled on weeks 0, 2, 4, 6, 8, 10 and 12. A total of 40 μl sera samples was incubated with 200 μl L-alanine and α-ketoglutamic acid for 30 min at 37°. Twenty minutes after the addition of 200 μl 2,4-dinitro-phenylhydrazine, 2 ml 0.4 N NaOH was added. UV absorptions were measured at 505 nm. ALT activities were calculated from the standard curve.

Statistics analysis

The experimental data were compared and analyzed by SPSS statistics software.

Results

Epitope selection and vector construction

The polytope plasmid, pMulE, was constructed as described in Method and used for DNA vaccination (Fig. 1). The polytope plasmid consisted of one universal T_h epitope, PADRE, and four continuous CTL epitopes of HBsAg (28, 29) (L^d-CTL epitope S_{28-39}, D^d-CTL epitope $S_{198-209}$ and two K^b-CTL epitopes $S_{172-191}$ and $S_{208-216}$). PADRE is a synthetic T_h epitope engineered by introducing anchor residues for the different DR motifs of MHC II into a polyalanine backbone (30) and the resulting peptide binds a variety of DR molecules as well as certain mouse class II alleles, including I-A^b, I-E^d and I-E^k. Every epitope was separated from each other with one lysine (Fig. 1). Such a spacer had previously been shown to minimize cleavage bias that may vary at epitope junctions due to the naturally occurring C- and N-terminal sequences of the minimal epitopes themselves (31). The presence of lysine or arginine at the carboxyl-terminus flanking residue was most frequently associated with strong CTL responses (31).

Polytope plasmids induced CTL responses in vivo

Groups of H-2^d BALB/c and H-2^b C57BL/6 mice ($n = 6$) were vaccinated by intramuscular (i.m.) injection with the pCI (100 μg), pS (100 μg) or pMulE (100 μg) vaccine plasmid, respectively. The polytope DNA vaccination induced vigorous CTL responses to the syngeneic HBsAg-expressing target cells in H-2^d and H-2^b mice; moreover, it resulted in a moderate but significant [using a two-way analysis of variance (ANOVA) of all the data, $P = 0.011$ in BALB/c mice, $P = 0.009$ in C57BL/6 mice] increase (averaging ≈22% in BALB/c, 24% in C57BL/6) in CTL responses compared with the single HBsAg DNA immunization (Fig. 2). The splenocytes from the mice vaccinated with the control plasmid had no significant specific target cell lysis.

To determine whether the polytope vaccination could induce CTL responses *in vivo* to the individual epitopes contained within the polytope plasmid, the splenocytes from each mouse were re-stimulated with the indicated peptide or endogenous processed HBsAg and used in the chromium release assays against the target cells sensitized with the same peptide. The pMulE-vaccinated BALB/c and C57BL/6 mice produced effectors specific for each of the two epitopes presented in

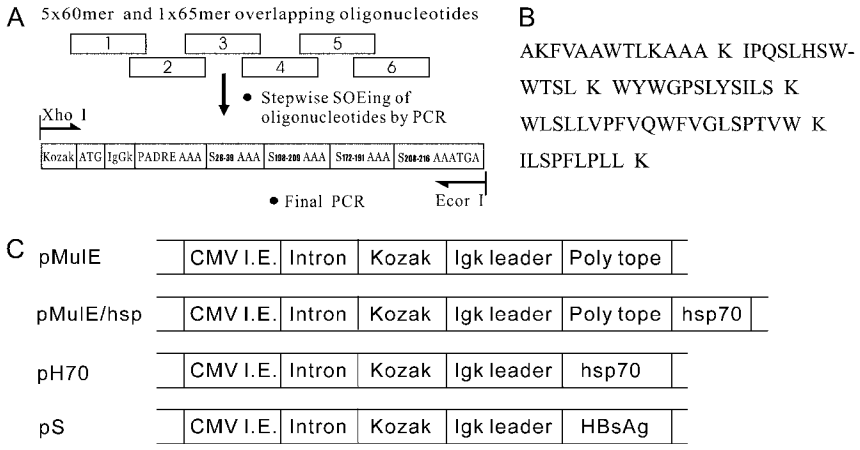


Fig. 1. Construction of vaccine plasmids for DNA vaccination. (A) The DNA sequence coding for the polytope antigen contained Kozak sequence, IgG κ leader sequence, PADRE sequence and four CTL epitopes. Each epitope was separated with AAA nucleotides encoding lysine. This polytope gene was made by joining six overlapping oligonucleotides using splicing by overlap extension and PCR and was cloned into pCI vector. So the polytope plasmid, pMulE, was generated. (B) Amino acid sequence of polytope antigen, including PADRE sequence, L^d-CTL epitope S_{28-39}, D^d-CTL epitope $S_{198-209}$ and two K^b-CTL epitopes $S_{172-191}$ and $S_{208-216}$. (C) Vaccine plasmids used for DNA vaccination were constructed based on pCI vector. The plasmid pMulE encoded polytope antigen, pMulE/hsp encoded hsp70-fused polytope antigen, pH70 encoded hsp70 and pS encoded HBsAg.

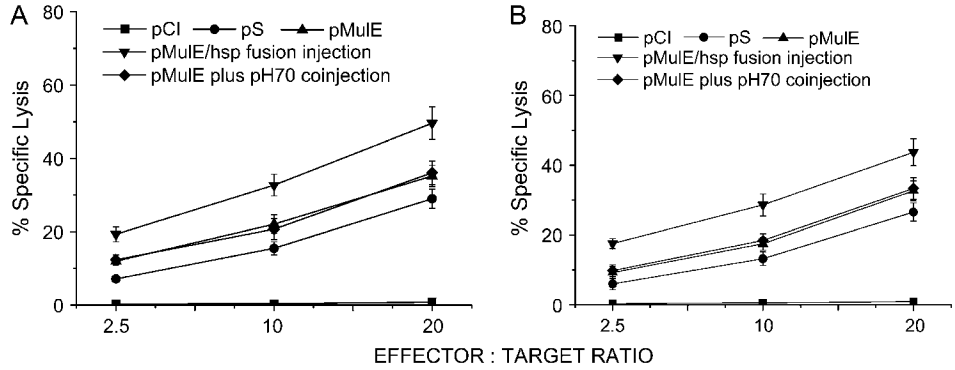


Fig. 2. CTL responses to HBsAg in BALB/c and C57BL/6 mice. Groups of BALB/c H-2^d and C57BL/6 H-2^b mice ($n = 6$) were vaccinated by i.m. injection with pCI (100 μg), pS (100 μg), pMulE (100 μg) or pMulE/hsp plasmid (100 μg) and co-injection with pMulE plus pH70 plasmids (100 μg plus 100 μg), respectively. The splenocytes from BALB/c (A) and C57BL/6 (B) mice were re-stimulated *in vitro* with the irradiated syngeneic HBsAg-expressing cells, respectively, and then were used in standard ^{51}Cr release assays against P815/S target cells (for BALB/c, H-2^d effectors) or EL4/S target cells (C57BL/6, H-2^b effectors). Specific lysis is expressed as percent lysis obtained from HBsAg-expressing target cells (P815/S or EL4/S) minus the percent lysis obtained using the same effector and parental target cells (P815 or EL4) ±SD. The percent lysis values obtained from the latter never exceeded 5%.

these two mouse strains (Fig. 3). These results demonstrated that polytope DNA vaccine induced independent MHC-restricted CTL responses to the multiple individual epitopes and viral antigen. Surprisingly, when re-stimulated with the irradiated syngeneic HBsAg-expressing P815/S cells, the pMulE vaccination elicited much weaker CTL responses to D^d-CTL epitope $S_{198-209}$ than those to L^d-CTL epitope S_{28-39} in BALB/c mice (Fig. 3B and D).

Polytope plasmid induced T_h proliferation

The induction of T_h is a crucial component of both humoral and cellular immune response. T_h secrete cytokines, such as IL-2, that play a fundamental role in the induction and the differentiation of B cell precursors into antibody-forming cells. The secretion of cytokines by T_h is also important in the differentiation and the maturation of CTLs. In our vaccine design, a universal helper CD4 T cell epitope, PADRE, was incorporated into the polytope plasmid as CD4 T_h activator. The immunogenicity of PADRE was tested by the T cell proliferation assay. When stimulated with the artificially synthesized PADRE peptide, the splenocytes from the vaccinated mice showed vigorous proliferation (Fig. 4A); moreover, IL-2 and IFN-γ were secreted by these splenocytes in the parallel cytokine assays (Fig. 4B). All these results indicated that CD4 T cell responses were elicited by the polytope vaccine.

CTL responses primed by polytope plasmid were mediated by hsp70

To improve the CTL responses, hsp70 molecule was used as a genetic adjuvant. The hsp-fused polytope plasmid and the hsp-expressing plasmid were constructed and named as pMulE/hsp and pH70, respectively (Fig. 1C). Groups of BALB/c

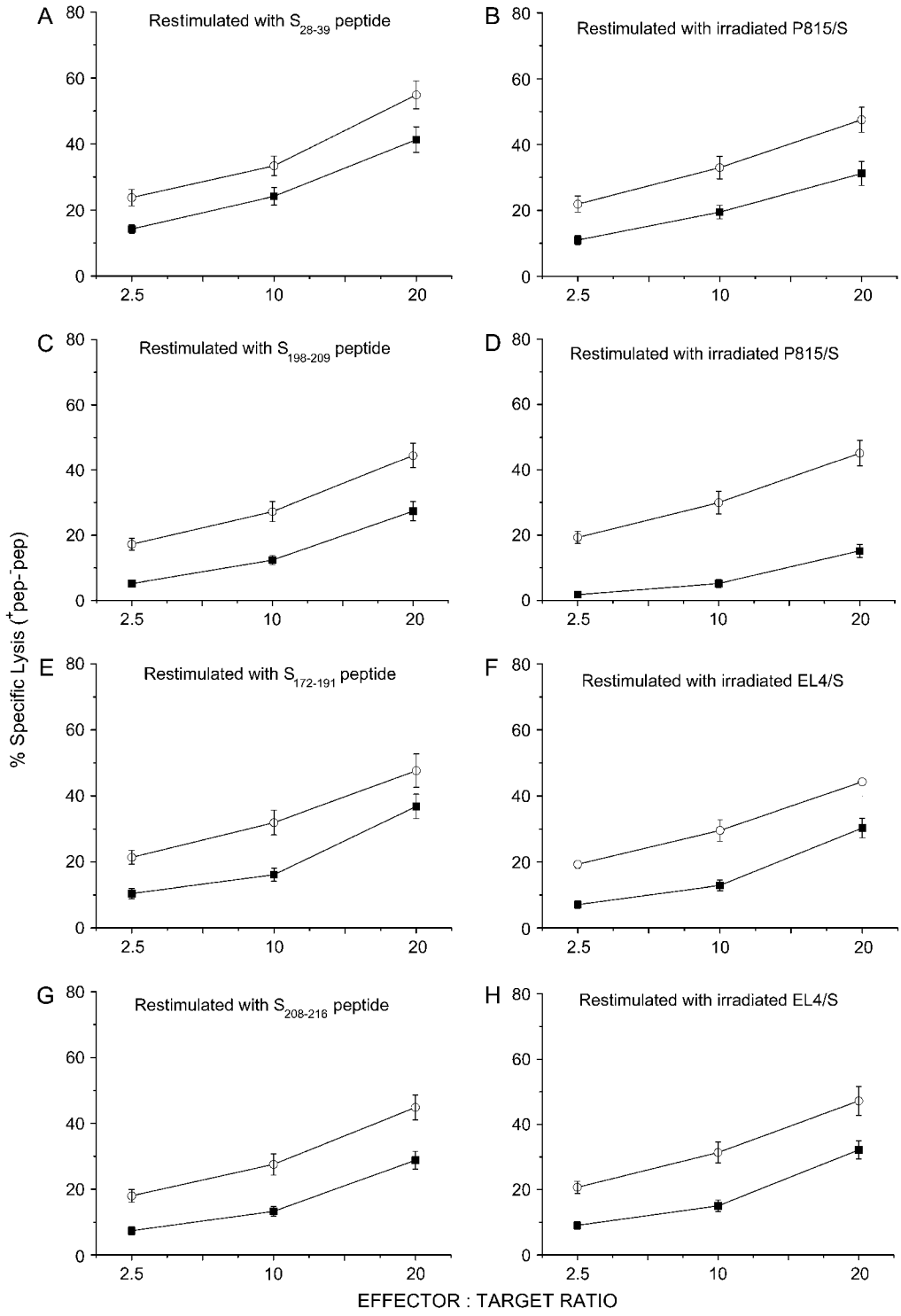


Fig. 3. CTL responses to each epitope in BALB/c and C57BL/6 mice. Groups of BALB/c H-2^d and C57BL/6 H-2^b mice ($n = 6$) were, respectively, vaccinated by i.m. injection with 100 μg pMulE plasmid (closed circle) and 100 μg pMulE/hsp plasmid (opened circle). The splenocytes from BALB/c (A–D) and C57BL/6 (E–H) mice were re-stimulated *in vitro* with the indicated CTL epitope peptides or the irradiated syngeneic HBsAg-expressing cells, respectively, and then were used in standard ^{51}Cr release assays against the relevant peptide-sensitized P815 target cells (for BALB/c, H-2^d effectors) or EL target cells (C57BL/6, H-2^b effectors). (A and B) against S_{28-39}-sensitized P815 cells, (C and D) against $S_{198-209}$-sensitized P815 cells, (E and F) against $S_{172-191}$-sentisitized EL4 cells and (G and H) against $S_{208-216}$-sensitized EL4 cells. Specific lysis is expressed as percent lysis obtained from peptide-sensitized target cells ($^{+}$pep) minus the percent lysis obtained using the same effector and target cells in the absence of peptide ($^{-}$pep) ±SD. The percent lysis values obtained from the latter never exceeded 5%.

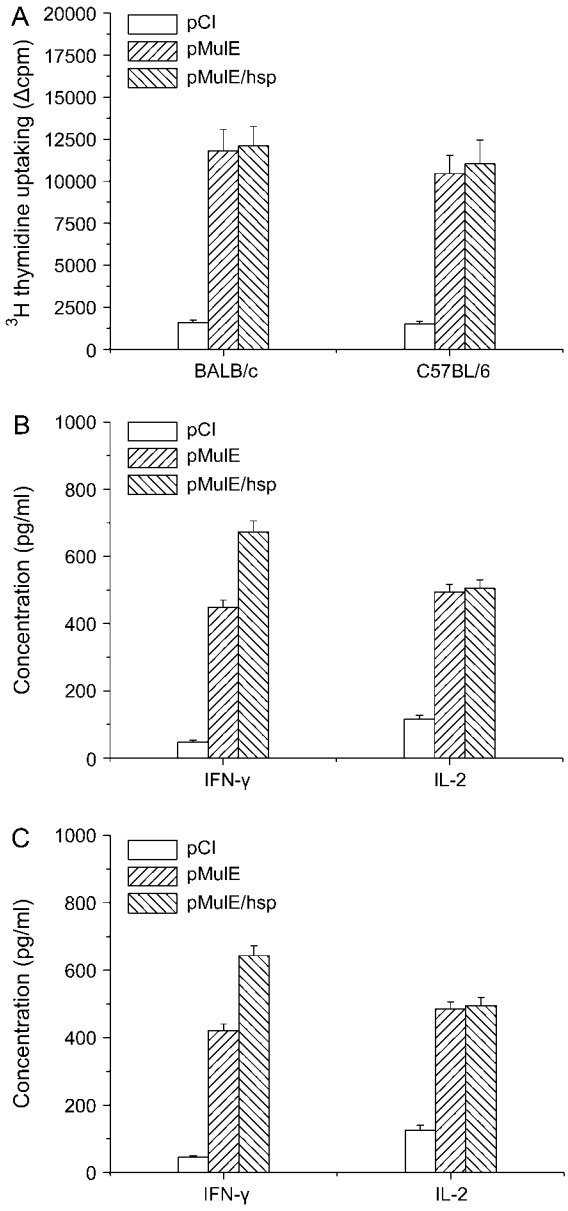


Fig. 4. T_h responses in BALB/c and C57BL/6 mice. Groups of BALB/c and C57BL/6 mice ($n = 6$) were vaccinated by i.m. injection with pCI (100 μg), pMulE (100 μg) or pMulE/hsp plasmid (100 μg), respectively. The splenocytes were re-stimulated *in vitro* with synthesized PADRE peptide in triplicate, and T_h responses were monitored. (A) T cell proliferation assay. After being re-stimulated for 3 days, the splenocytes from BALB/c and C57BL/6 mice were, respectively, used in standard [^{3}H]TdR incorporation assay and the results were expressed as Δ counts per minute corrected for background activity derived from the non-stimulated spleen cells. (B and C) Cytokine profile of proliferating T cells. The splenocytes from BALB/c (B) and C57BL/6 (C) mice were re-stimulated with PADRE peptide as above. The supernatants were collected, and then IL-2 and IFN-γ concentrations (pg ml^{-1}) were determined by ELISA. The data were presented as mean ± SD for six animals.

and C57BL/6 mice ($n = 6$) were vaccinated by i.m. injection with pMulE/hsp plasmid (100 μg) and i.m. co-injection with pMulE plus pH70 (100 μg plus 100 μg), respectively. In both BALB/c and C57BL/6 mice, the pMulE/hsp vaccination elicited stronger CTL responses to HBsAg than the pMulE vaccination (Fig. 2). It had an average 40% increase ($P = 0.005$) in BALB/c mice and an average 34% increase ($P = 0.004$) in C57BL/6 mice (using a two-way ANOVA of all the data) in CTL responses to HBsAg. Moreover, the CTL responses to each epitope were also significantly enhanced (using a two-way ANOVA of all the data, $P < 0.05$) when immunized with the hsp-fused polytope plasmid in H-2^d and H-2^b mice (Fig. 3). However, when co-injecting the pMulE plasmid with the pH70 plasmid, neither BALB/c nor C57BL/6 mice showed the distinct increased CTL responses (Fig. 2). Furthermore, the splenocytes from the mice immunized with the pMulE/hsp secreted much more IFN-γ (Student's *t*-test, $P < 0.05$, $n = 6$) than those from the mice immunized with the pMulE in the cytokine secretion assay (Fig. 4B), but these splenocytes only showed little changes in T cell proliferation assay (Fig. 4A) and the secretion of IL-2 had no significant change. These results indicated that the enhanced CTL responses were not due to PADRE-specific CD4 T_h responses.

Polytope vaccination resulted in the clearance of HBsAg and the down-regulation of HBV DNA replication in a HBV transgenic mouse model

It is known that the clearance and the control of HBV infection are related closely with the host cellular immune responses. Therefore, the potential therapeutic effect of the polytope vaccine was evaluated in the HBV transgenic mice. This mouse model was established by embryonic injection and was tolerant to HBV antigen. Following expression of HBV, viral particles were assembled in liver cells and secreted into blood. Therefore, it was used as a chronic HBV carrier model. Groups of mice ($n = 6$) were vaccinated by i.m. injection with the pCI (100 μg), pS (100 μg), pMulE (100 μg) or pMulE/hsp plasmid (100 μg), respectively. After the vaccinations, HBsAg significantly dropped in the sera since the first week after immunization in the pS, pMulE and pMulE/hsp groups; moreover, HBsAg were undetectable after 10 weeks (Fig. 5A). Interestingly, the polytope vaccinations, both the pMulE and the pMulE/hsp, resulted in the more rapid down-regulation of HBsAg ($P < 0.05$, using two-way ANOVA of all the data) than the single HBsAg DNA vaccination. However, anti-HBsAg antibody was not observed by ELISA or western blotting. Furthermore, HBV DNA in the serum was monitored. HBV DNA was distinctly down-regulated and kept at an extremely low level (Fig. 5B). After 6 months, HBsAg was still undetectable and HBV DNA still kept at this low level.

Polytope vaccination induced CTL and T_h responses in HBV transgenic mice

Since the polytope vaccinations resulted in the significant clearance of HBsAg and down-regulation of HBV DNA, cellular immune responses were checked in the HBV transgenic mice. We found that T cell responses in the transgenic mouse model were similar to those in the BALB/c mouse model. The pMulE vaccination resulted in a moderate but significant (using a two-way ANOVA of all the data, $P = 0.007$) increase (averaging ≈30%) in CTL responses to HBsAg-expressing target cells compared with the single HBsAg DNA vaccination in HBV transgenic mice (Fig. 6). Moreover, the

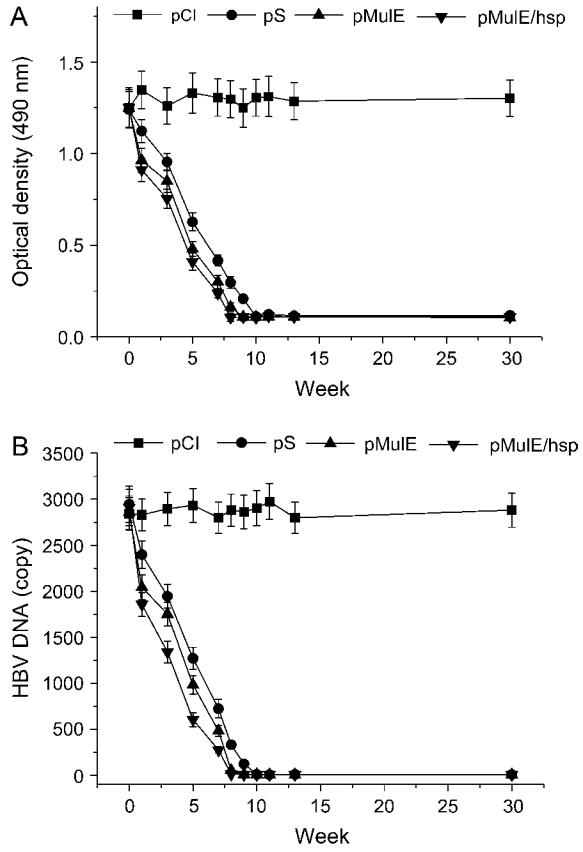


Fig. 5. Serology assays in HBV transgenic mice. Groups of H-2^d HBV transgenic mice ($n = 6$) were vaccinated by i.m. injection with pCI (100 μg), pS (100 μg), pMulE (100 μg) or pMulE/hsp plasmid (100 μg), respectively. Mice were bled at different time points after the first injection. Sera were obtained and assayed for the presence of HBsAg (A) by ELISA and HBV DNA (B) by quantitative real-time PCR. The data were presented as mean ± SD for six animals per time point.

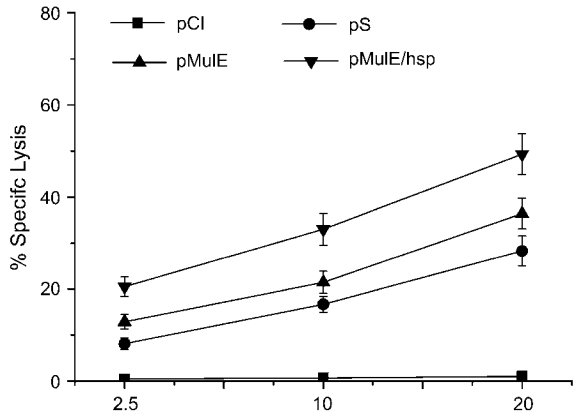


Fig. 6. CTL responses to HBsAg in HBV transgenic mice. Groups of H-2^d HBV transgenic mice ($n = 6$) were vaccinated by i.m. injection with pCI (100 μg), pS (100 μg), pMulE (100 μg) or pMulE/hsp plasmid (100 μg), respectively. The splenocytes were re-stimulated *in vitro* with the irradiated syngeneic HBsAg-expressing cells, and then were used in standard ^{51}Cr release assays against P815/S target cells. Specific lysis is expressed as percent lysis obtained from HBsAg-expressing target cells (P815/S) minus the percent lysis obtained using the same effector and parental target cells (P815) ±SD. The percent lysis values obtained from the latter never exceeded 5%.

pMulE/hsp vaccination induced stronger CTL responses to viral antigen and each epitope ($P < 0.05$ using two-way ANOVA of all the data) than the pMulE vaccination (Figs 6 and 7). In T_h responses assay, the pMulE and the pMulE/hsp vaccinations caused the significant T_h proliferations and the secretion of IFN-γ and IL-2 (Fig. 8). Although the splenocytes from the pMulE/hsp vaccinated mice produced more IFN-γ (Student's *t*-test, $P < 0.05$, $n = 6$) than those from the pMulE-vaccinated mice, no significant change was observed in the secretion of IL-2 and T_h proliferation assays.

Liver injury assay

Liver injury was evaluated by checking ALT activities in the sera of the HBV transgenic mice. ALT activity rose after the pMulE/hsp vaccination, and then returned to the normal level and sustained at these levels until the 13th week (Fig. 9). After 6 months, ALT still was kept at this level (data not shown).

Discussion

From the history of HBV infection, it is known that the key for therapy of chronic HBV infection is to induce specific and vigorous cellular immune responses, including CD8$^+$ CTL and CD4$^+$ T_h responses. Epitope-based DNA vaccination represents a new therapy strategy for viral infection because of its excellent ability to induce T cell responses. DNA vaccine coding multiple continuous CTL epitope can induce specific CTL responses to individual epitope from the different antigens (32), and DNA vaccine coding several T_h epitopes can prime specific T_h responses to each epitope (33). In our research, polytope DNA vaccine containing four CTL epitopes and one universal T_h epitope induced both T_h and CTL responses to each epitope, and it induced much stronger CTL responses to HBsAg than the single-antigen DNA vaccination. Antibody responses against the polytope antigen were not detected in BALB/c or C57BL/6 mice vaccinated by the i.m. injection with the polytope plasmids. Western blotting assay with sera from these mice did not detect a green fluorescent protein (GFP)-fused polytope antigen, while rabbit polyclonal antibody specific for GFP, sc-8334, did recognize the fusion antigen by these assays (data not shown). Surprisingly, we found that the CTL response to $S_{198-209}$ was much weaker than that to S_{28-29} in BALB/c mice; moreover, it was only significantly primed at the higher effector/target ratio. R. Schirmbeck found that L^d-restricted T cell response (S_{28-39}) to HBsAg efficiently suppressed T cell priming to multiple D^d-, K^d- and K^b-restricted HBsAg epitopes ($S_{198-209}$, $S_{172-191}$ and $S_{208-216}$) in H-2$^{d\times b}$ mating mice, but the suppression of L^d-restricted epitope did not hinder the protection function of other epitopes *in vivo* (34). Therefore, this research provided the reasonable explanation for our results. Nevertheless, this suppression did not affect the CTL responses to K^d- and K^b-restricted epitope in C57BL/6 mice since L^d-restricted epitope was not primed in H-2^b mice.

The previous research showed that the immune responses to a DNA vaccine could be regulated by co-delivery of various cytokine genes (35). These cytokines modulated the immune responses by favoring the development of T_h1 versus T_h2.

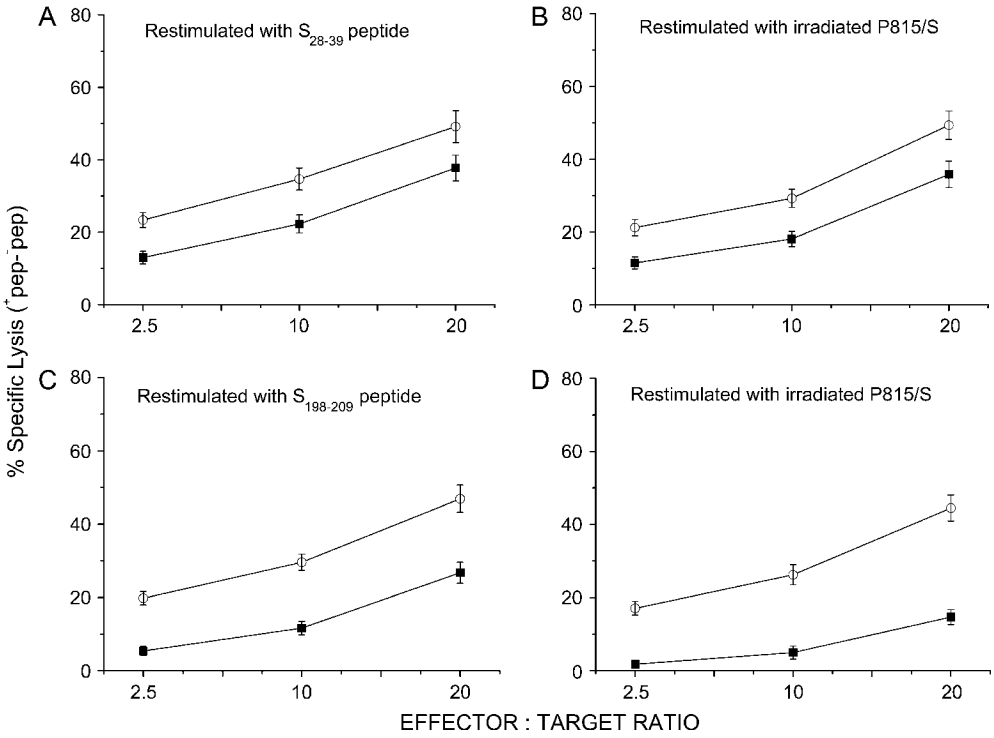
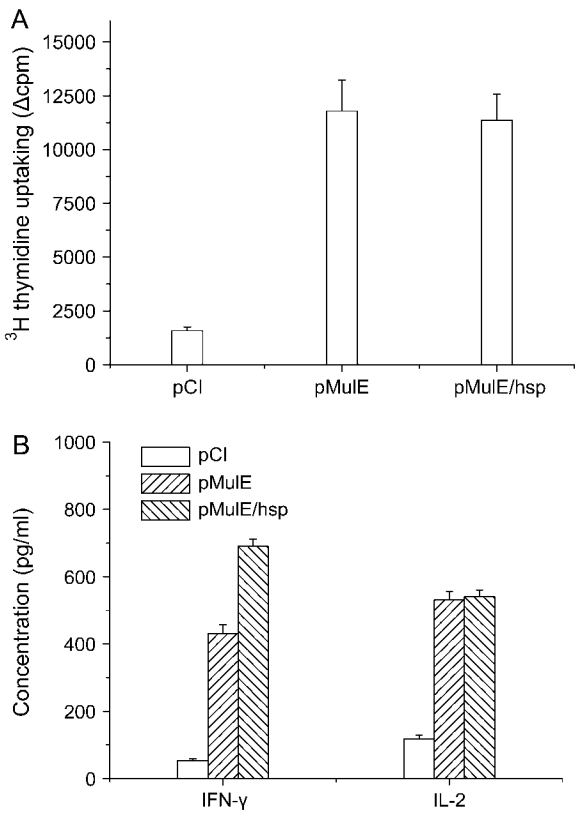


Fig. 7. CTL responses to each epitope in HBV transgenic mice. Groups of HBV transgenic mice ($n = 6$) were, respectively, vaccinated by i.m. injection with 100 μg pMulE plasmid (closed circle) or 100 μg pMulE/hsp plasmid (opened circle). The splenocytes were re-stimulated *in vitro* with the indicated CTL epitope peptides or the irradiated syngeneic HBsAg-expressing cells, respectively, and then were used in standard ^{51}Cr release assays against the relevant peptide-sensitized P815 target cells. (A and B) against S_{28-39}-sensitized P815 cells and (C and D) against $S_{198-209}$-sensitized P815 cells. Specific lysis is expressed as percent lysis obtained from peptide-sensitized target cells ($^{+}$pep) minus the percent lysis obtained using the same effector and target cells in the absence of peptide ($^{-}$pep) ±SD. The percent lysis values obtained from the latter never exceeded 5%.



Therefore, this method provided a strategy to improve the DNA vaccine's prophylactic and therapeutic efficacy. In our study, hsp70 molecule was used as genetic adjuvant to improve T cell responses since it had both cytokine and chaperone functions. When hsp70 gene was fused to the polytope plasmid, it enhanced the CTL responses in both BALB/c and C57BL/6 mice. However, co-injecting the polytope plasmid with the pH70 plasmid did not result in significant changes in CTL responses. Thus, hsp70 mediated CTL responses by a pathway that was different from cytokines. It was known that hsp70 and hsp60 shared the common receptors that were expressed on human monocyte-derived dendritic cells (36). In H-2^{d} and H-2^{d} mice models, all hsp utilized the CD91R to be internalized by antigen-presenting cells (APCs), and the

Fig. 8. T_h responses in HBV transgenic mice. Groups of HBV transgenic mice ($n = 6$) were vaccinated by i.m. injection with pCI (100 μg), pMulE (100 μg) or pMulE/hsp plasmid (100 μg), respectively. The splenocytes were re-stimulated *in vitro* with synthesized PADRE peptide in triplicate, and T_h responses were monitored. (A) T cell proliferation assay. After being re-stimulated for 3 days, the splenocytes from transgenic mice were, respectively, used in standard [^{3}H]TdR incorporation assay and the results were expressed as Δ counts per minute corrected for background activity derived from the non-stimulated spleen cells. (B) Cytokine profile of proliferating T cells. The splenocytes were re-stimulated with PADRE peptide as above. The supernatants were collected, and then IL-2 and IFN-γ concentrations (pg ml^{-1}) were determined by ELISA. The data were presented as mean ± SD for six animals.

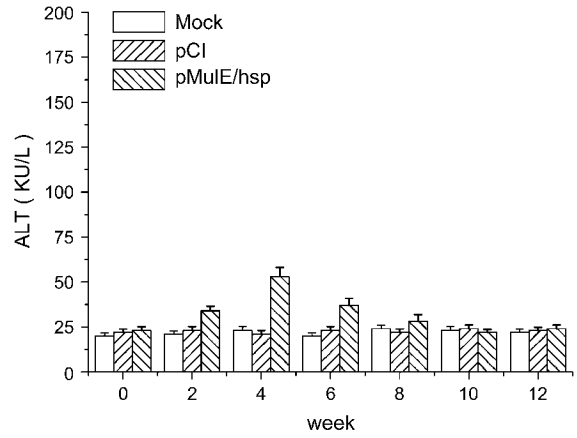


Fig. 9. ALT assay. Liver injury was evaluated by ALT activity assay. Mice were bled on the 0th, 2nd, 4th, 6th, 8th, 10th and 30th week after the first injection. Sera were prepared and stored at −70°C for assays. ALT activity was monitored. The values were calculated by the standard curve of pyruvic acid. The results were presented as mean ± SEM for six animals per time point.

complexes of peptides with hsp90, calreticulin and hsp70 were also taken up by macrophages and dendritic cells and represented by MHC class I molecules (37). Therefore, we supposed that the hsp70-fused polytope antigen was specially internalized by receptor-mediated endocytosis and APCs presented the hsp-associated peptides, via their cell-surface MHC class I molecules, to $CD8^+$ T cells; thus, antigen-presenting function was improved. Interestingly, we found that hsp70 not only regulated the CTLs to HBsAg but also enhanced the CTL response to D^d-restricted epitope that was suppressed by L^d-restricted CTL epitope in H-2^d mice. This result was coincident with the previous study (27). Therefore, hsp70 was an intrinsic adjuvant molecule for polytope DNA vaccine.

Furthermore, we evaluated potential therapeutic effects of the polytope DNA plasmids in a HBV transgenic mouse model. As expected, the polytope vaccinations resulted in a long-term clearance of HBsAg. Moreover, hsp70 improved this therapeutic effect as an adjuvant. Nevertheless, HBV DNA was not cleared completely. The trace amount viral gene was still checked out by quantitative PCR, while it was undetectable by northern blotting (data not shown). It suggested that the transgene expression was not extinguished but rather controlled and HBV-specific CTLs suppressed HBV gene expression and replication in the transgenic mice. These results were coincident with the previous research (38). In addition, we found that no obvious liver injury occurred following DNA immunization. Therefore, the viral clearance was mainly due to the non-cytopathic and lymphokine-based anti-viral mechanism rather than the destruction of infected cell.

HBV has several variations and CTL epitopes not only exist in the surface antigen but also in the core antigen and the polymerase. Although HBV variants with mutations in dominant T cell epitopes might arise during acute hepatitis B infection, they typically remain in low abundance and do not necessarily affect clinical recovery (39). Herein, we chose four CTL epitopes and one T_h epitope to design the polytope DNA vaccine, and found that it primed the specific CTL to each epitope and viral antigen in the different MHC-restricted mice models. The polytope vaccine inhibited viral gene replication and cleared the circulating viral antigen in the HBV transgenic mouse model. Furthermore, hsp70 improved the vaccine's ability to induce specific CTL responses. According to the studies and data presented here, we believe that the polytope DNA vaccine fused with hsp gene may provide an effective therapeutic strategy for chronic HBV carriers.

Acknowledgements

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Abbreviations

ALT	alanine aminotransferase
ANOVA	analysis of variance
APC	antigen-presenting cell
GFP	green fluorescent protein
[^{3}H]TdR	[^{3}H]thymidine
HBV	hepatitis B virus
HbsAg	hepatitis B virus surface antigen
hsp	heat shock protein
i.m.	intramuscular
PADRE	Pan DR epitope

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Appendix B

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Vaccine. 2000 Nov 22;19(7-8):764-78.

Induction of antigen-specific CD8+ T cells, T helper cells, and protective levels of antibody in humans by particle-mediated administration of a hepatitis B virus DNA vaccine.

Roy MJ, Wu MS, Barr LJ, Fuller JT, Tussey LG, Speller S, Culp J, Burkholder JK, Swain WF, Dixon RM, Widera G, Vessey R, King A, Ogg G, Gallimore A, Haynes JR, Heydenburg Fuller D.

PowderJect Vaccines Inc., 585 Science Drive, Madison, WI 53711, USA.

Abstract

A DNA vaccine against the hepatitis B virus (HBV) was evaluated for safety and induction of immune responses in 12 healthy, hepatitis-naïve human volunteers using the needle-free PowderJect system to deliver gold particles coated with DNA directly into cells of the skin. Three groups of four volunteers received three administrations of DNA encoding the surface antigen of HBV at one of the three dose levels (1, 2, or 4 microg). The vaccine was safe and well tolerated, causing only transient and mild to moderate responses at the site of administration. HBV-specific antibody and both CD4+ and CD8+ T cell responses were measured before and after each immunization. All the volunteers developed protective antibody responses of at least 10 mIU/ml. In volunteers who were positive for the HLA class I A2 allele, the vaccine also induced antigen-specific CD8+ T cells that bound HLA-A2/HBsAg(335-343) tetramers, secreted IFN-gamma, and lysed target cells presenting a hepatitis B surface antigen (HBsAg) CTL epitope. Enumeration of HBsAg-specific T cells producing cytokine indicated preferential induction of a Type 1 T helper cell response. These results provide the first demonstration of a DNA vaccine inducing protective antibody titers and both humoral and cell-mediated immune responses in humans.

PMID: 11115698 [PubMed - indexed for MEDLINE]

Publication Types, MeSH Terms, Substances

LinkOut - more resources

DNA Injection in Combination with Electroporation: a Novel Method for Vaccination of Farmed Ruminants

S. Tollefsen,* M. Vordermeier,† I. Olsen,‡ A. K. Storset,§ L. J. Reitan,‡ D. Clifford,¶ D. B. Lowrie,** H. G. Wiker,†† K. Huygen,‡‡ G. Hewinson,† I. Mathiesen§§ & T. E. Tjelle§§

*Immunological Institute, Rikshospitalet University of Oslo, Oslo, Norway; †Veterinary Laboratories Agency, TB Research Group, Surrey, UK; ‡National Veterinary Institute; §Norwegian School of Veterinary Science, Oslo, Norway; ¶Animal Services Unit New Haw, Surrey; **National Institute for Medical Research, London, UK; ††Norwegian Institute of Public Health, Department of Environmental Immunology, Oslo, Norway; ‡‡Mycobacterial Immunology, Pasteur Institute, Brussels, Belgium; and §§Physiological Institute, University of Oslo, Oslo, Norway

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Correspondence to: Dr S. Tollefsen, Institute of Immunology, University of Oslo, Rikshospitalet, 0027 Oslo, Norway. E-mail: stig.tollefsen@labmed.uio.no

Abstract

Injection of plasmid DNA encoding antigens into rodents followed by electroporation improved the immune response when compared with injection without electroporation (Widera *et al.* J Immunol 2000;164:4635–40; Zucchelli *et al.* J Virol 2000;74:11598–607; Kadowaki *et al.* Vaccine 2000;18:2779–88). The present study describes the extension of this technology to farm animals, by injecting plasmid DNA encoding mycobacterial antigens (MPB70, Ag85B and Hsp65) into the muscles of goats and cattle using two different types of electrodes, both allowing DNA delivery at the site of electroporation. The animals were vaccinated under local anaesthesia without any observed immediate or long-term distress or discomfort, or any behavioural signs of muscle damage or pathological changes after the electroporation. DNA-injected and electroporated goats showed increased humoral response after the primary vaccination when compared with nonelectroporated animals. Improved T-cell responses following electroporation were observed in *hsp65* DNA-vaccinated cattle. DNA injection with or without electroporation did not compromise the specificity of the tuberculin skin test. In conclusion, a protocol applying *in vivo* electroporation free of side effects to farmed ruminants was established. In addition, we show that DNA vaccination in combination with electroporation can improve the primary immune responses to the encoded antigens.

Introduction

It is estimated that more than 50 million cattle are infected with *Mycobacterium bovis* worldwide, resulting in economic losses of approximately $3 billion annually [1]. Bovine tuberculosis is also a health issue in farmed goats. In Great Britain (GB), control of bovine tuberculosis is based on a test and slaughter strategy that relies on the intradermal tuberculin test to identify infected animals. However, this strategy has failed to eradicate the disease completely, and since mid-1980s, a dramatic rise in the incidence of tuberculosis in cattle has been reported [2]. The urgency for new and improved cattle vaccines and diagnostic reagents has been highlighted in a recent independent scientific review of the situation in GB [2]. The only vaccine currently available, *M. bovis* bacille Calmette–Guérin (BCG), is associated with a highly variable and unpredictable efficacy both in humans and in cattle. As it also compromises the specificity of the intradermal tuberculin test [3, 4], vaccination of cattle with BCG cannot be considered a valid option at present in countries wishing to continue with existing test and slaughter-based control programmes. Recently, we reported that vaccination of cattle with plasmid DNA encoding mycobacterial antigens like MPB70 or MPB83 did not interfere with the intradermal tuberculin test, suggesting that this diagnostic procedure could continue in animals immunized by DNA vaccination [5].

Protection against tuberculosis using DNA vaccines has been demonstrated in small animal models for a range of different mycobacterial antigens. For example, mice immunized with plasmid DNA encoding mycobacterial Hsp65 antigen were protected against *Mycobacterium tuberculosis* challenge [6]. Furthermore, this *hsp65* DNA vaccine and also an *mpt70* DNA vaccine were shown to reduce bacterial loads in mice infected with *M. tuberculosis*. Moreover, therapeutic administration of the vaccine in a Cornell-type model of relapse resulted in sterilizing immunity in some animals [7]. MPB70 is a serodominant

antigen in *M. bovis*-infected cattle and is well recognized by T lymphocytes from infected animals but not from BCG-vaccinated cattle [8, 9]. Plasmid DNA encoding MPB70 induced T helper 1 (Th1)-type immune responses in vaccinated cattle [5]. The fibronectin-binding protein antigens 85A and 85B (Ag85) are prominent secreted antigens found in the culture filtrates of *M. tuberculosis* and *M. bovis* BCG [10, 11], and as such, they are apparent candidates for vaccination against tuberculosis. In the form of DNA vaccines, Ag85A and Ag85B have demonstrated highly protective effect against tuberculosis infection in mouse and guinea-pig models [12–14].

Over the last years, studies describing DNA injection in combination with electroporation in skeletal muscle have demonstrated that this method induced a strong and lasting immune response [15–18]. In addition, it has been shown that the immune response is practically unaffected by a 10- to 100-fold reduction of DNA dose when electroporation is applied immediately after injection [16, 19]. In the present study, we demonstrate the applicability of electroporation for vaccination of animal species like domesticated ruminants.

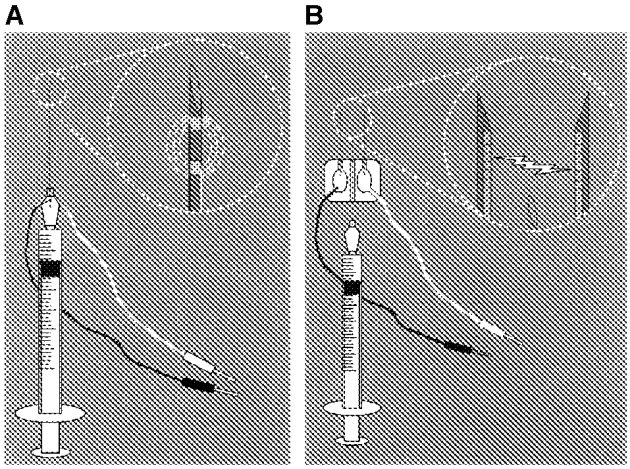

Figure 1 The electrodes used for DNA injection and electroporation. Panel A shows the electrode used in goats, a modified syringe for delivery of DNA, with two silver electrodes for generation of an electrical field. Panel B shows the electrode used in cattle, made of two syringes with gold electrodes for generation of an 'array-like' electrical field. The electrodes had fixed positions in polycarbonate, with a hole for injection of DNA through a standard syringe delivering the DNA at the right depth between the electrodes. The electrodes were connected to a Hear 6 bp stimulator (goats) or a generator made by INOVIO, Norway (cattle) for generation of the electrical field.

Materials and methods

Animals. Cattle: Three pairs of twins (females, about 2 years old, Friesian or Friesian crosses) were obtained from herds free of bovine tuberculosis and kept in the Animal Services Unit at VLA Weybridge. *Goats*: Six male outbred goats, 6 weeks of age, were obtained from the Agricultural University of Norway (Ås, Norway). For comparison, blood samples were analysed from three nonvaccinated goats living outdoor on the institute ground. All goats in this study were from the same herd, weight and age-matched, and treated in accordance with Norwegian rules for animal experiments.

DNA vaccines. The following plasmids were used: *Mycobacterium leprae hsp65* (pCMV4.65) [6], *M. tuberculosis ag85b* (V1J.ns-tPA.85B) [20] and *mpb70* (pCMV70) [7].

Antigens. Bovine (PPD-B) and avian (PPD-A) tuberculins were obtained from the Tuberculin Production Unit at the Veterinary Laboratories Agency-Weybridge and used in culture at 10 μg/ml. Recombinant *M. tuberculosis* Hsp65 was purchased from Lionex Ltd (Braunschweig, Germany) and used at 5 μg/ml in tissue culture. The secreted mycobacterial proteins MPB70 [10] and Ag85B [21] were purified from culture supernatant of *M. bovis* BCG Tokyo and BCG Copenhagen, respectively, grown in the wholly synthetic Sauton medium [10, 11]. PPD-P isolated from *Mycobacterium avium* ssp. *paratuberculosis* was produced at the National Veterinary Institute, Oslo, Norway.

Electrodes, electroporator and electroporation. For electroporation, modified syringes, as shown in Fig. 1A (goats) and Fig. 1B (cattle), were used, both of which allowed electroporation at the site of DNA delivery. For electroporation of cattle, an Inovio generator was used (INOVIO, Oslo, Norway). For electroporation of goats, a Hear 6 bp stimulator (Frederick Hear, Bowdoinham, ME, USA) was used [22]. The electric stimulation given to the muscle in both cases consisted of 1000 short pulses (200 μs positive and 200 μs negative) at 1000 Hz delivered in trains with 2 s between each train (five to 10 trains). The electric field strength varied with the resistance in the tissue of each animal, giving approximately 150–200 V/cm (Table 1).

Anaesthetics and experimental procedure. Three pairs of twin cows were divided into two groups containing one sibling in each group. Twin cattle are homoeopoetic chimeras, because of the anastomoses forming between the placentas, leading to an exchange of homoeopoetic stem cells and thus the best control for each other. Animals were sedated with intravenous injection of Rompun 2% (Bayer plc, Bury St. Edmunds, UK) (approximately 0.05 mg/kg body weight). Animals to be treated by electroporation were given local anaesthesia (Licaine 2%, Animal Care, Dunnington, York, UK), which was applied by infiltrating 20 ml of the anaesthetic subcutaneously and intramuscularly (i.m.) in an arc dorsal to the DNA injection and electroporation site in the middle third of the neck, approximately 10 cm below the nuchal ligament. DNA (pCMV4.65, 1 mg) was then injected into the middle part of the neck, followed by electroporation at weeks

Table 1 Electroporation and vaccination protocols applicable to large ruminants

	Goats	Cattle
Sedation	Rompun (2%) at 0.2 mg/kg, i.m.	Rompun (2%) at 0.05 mg/kg, i.m.
Anaesthetics	First treatment: 5 mg/kg Ketalar (total) Second/third treatment: 50 mg Xylocain (local)	20 ml of Licaine 2% (400 mg)
Plasmid DNA (dose per vaccination)	pCMV70, V1Jns-tPA.85B (2×50 μg of each plasmid/goat (first two treatments)) (2×90 μg of each plasmid/goat (third treatment))	pCMV4.65 (1 mg/ml/cow, divided on two to four injections)
Electrode	Combined single-needle syringe (Fig. 1A)	Separate double-needle electrodes (Fig. 1A)
Electroporation	Field strength: 150–200 V/cm; pulses: 200 μs positive, 200 μs negative Frequencies of pulses: 1000 Hz; number of 1000 pulses: 5–10	
Vaccination schedule	Weeks 0, 11 and 17	Weeks 0 and 3

A detailed description of goat and cattle sedation, anaesthetics, plasmid DNA dose, electrodes used and the electroporation procedure is given. The vaccination schedule indicates at which time points the animals were vaccinated. i.m., intramuscular.

0 and 3. For details of, e.g., electroporation conditions, see Table 1. The animals were observed for any signs of immediate or long-term distress or discomfort, or any behavioural signs of muscle damage. At the end of the experiment, animals were killed by lethal injection and the DNA injection sites were examined for pathological changes.

All six goats were given Rompun 2% (Bayer plc) (i.m.) as a sedative and analgeticum (0.2 mg/kg body weight), and further three of the six were given Ketalar (Pfizer Inc, New York, NY, USA) (5 mg/kg body weight) for total anaesthesia during the first DNA injection or given Xylocain (AstraZeneca, London, UK) (50 mg) as a local anaesthetic (boost injections) i.m. about 5–15 min before DNA injection and electroporation. A mixture of pCMV70 (100 μg) and V1J.ns-tpa.85B (100 μg) was injected into the right gastrocnemius muscle, and three of the animals were electroporated at the site of injection. After the treatment, all goats were given the antisedative Atipamezol, α_2-antagonist (Orion Pharma, Espoo, Finland) (0.05 mg/kg body weight), to avoid ruminal disorders. Boost injections were given at week 11 (200 μg DNA injected) and 17 (360 μg DNA injected). At week 22, the goats were euthanized, and the popliteal and subiliac lymph nodes from both sides of the animals in addition to the spleen were removed for cellular analyses.

Separation of mononuclear cells from blood, lymph nodes and spleen. Cattle: Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by Histopaque-1077 (Sigma, St Louis, MO, USA) gradient centrifugation and cultured in RPMI-1640 supplemented with 5% CPSR-1 (controlled process serum replacement type 1, Sigma Aldrich, Poole, UK), nonessential amino acids (Sigma Aldrich), 5×10^{-5} M 2-mercaptoethanol, 100 U/ml of penicillin and 100 μg/ml of streptomycin sulphate. Superficial cervical lymph nodes were removed 4 days after the injection of the skin-test reagents, and single-cell suspensions were prepared by the passage of tissue portions through metal tea strainers followed by Histopaque-1077 centrifugation, as described above. Cells were then suspended in RPMI-1640 supplemented as described above for PBMCs.

Goats: Single-cell suspensions were prepared by the passage of tissue portions from lymph nodes and spleen through a modified metal garlic press and further isolated and separated by Lymphoprep (Nycomed Amersham, Oslo, Norway) gradient centrifugation and cultured in RPMI-1640 supplemented with 10% fetal calf serum (FCS). The remaining erythrocytes were lysed with 0.14 M NH_4Cl in a Tris–HCl buffer, pH 7.2.

Lymphocyte transformation assay. PBMCs (cattle) (2×10^5/well in 0.2 ml aliquots) were cultured in triplicates for 6 days in flat-bottom 96-well microtitre plates in the presence of an antigen at 37 °C and 5% CO_2 and radiolabelled during the last 16–20 h of culture with 37 kBq of [^{3}H]-thymidine/well (Amersham, Amersham, UK), harvested onto glass-fibre filters and counted in a scintillation counter (TopCount, Packard, Pangborne, UK). Positive responses are defined by a stimulation index (SI) (cpm with antigen/cpm without antigen) ≥ 3 [23–25]. Average [^{3}H]-thymidine incorporation in unstimulated lymphocytes in the experiments shown was 1750 ± 296 cpm (range: 517–5461 cpm).

Interferon-γ enzyme-linked immunospot assay [8]. A direct enzyme-linked immunospot (ELISPOT) assay was established by modifying the protocol described for indirect ELISPOTs by van Drunen *et al.* [26]. Briefly, ELISPOT plates (Immobilon-PVDF membranes, Millipore, Molsheim, France) were coated overnight at 4 °C with the bovine IFN-γ-specific monoclonal antibody 2.2.1. Unbound antibody was removed by washing, and the wells were blocked with 10% FCS in AIM-V medium (Life Technologies, Paisley, Scotland, UK). PBMCs ($2–5 \times 10^5$/well suspended in AIM-V/2% FCS) were then added and cultured at 37 °C and 5% CO_2 in a humidified incubator for 24 h. Spots were developed with a rabbit antiserum specific for bovine IFN-γ, followed by incubation with an alkaline phosphatase-conjugated monoclonal antibody specific for rabbit immunoglobulin G (IgG)

(Sigma Aldrich). Both the monoclonal antibody 2.2.1 and the rabbit antibovine IFN-γ serum were kindly supplied by Dr D. Godson (VIDO, Saskatoon, Saskatchewan, Canada). The spots were visualized with BCIP-NBT substrate (Sigma Aldrich), and responses of spot-forming cells (SFCs) >10 were deemed positive.

Detection of antigen-specific antibodies by enzyme-linked immunosorbent assay. High-bind microtitre (Costar) plates were coated with purified native protein in the following concentrations: Ag85B and MPB70, 0.5 μg/well; and Hsp65, 0.1 μg/well. Serial dilutions of serum samples were then added to the plates, and enzyme-linked immunosorbent assay (ELISA) was performed as described previously [5]. IgG was detected using horseradish peroxidase (HRP)-conjugated sheep antibovine IgG antisera (Serotec, Oxford, UK) or HRP-conjugated mouse monoclonal anti-goat IgG (clone GT-34, Sigma).

Tuberculin skin test. Comparative intradermal tuberculin skin tests were performed as specified in the EEC Directive 80/219EEC, Amending Directive 64/422/EEC, Annexe B [27]. The antigen injected was bovine tuberculin (PPD-B), avian tuberculin (PPD-A), 100 μg each, or recombinant *M. tuberculosis* Hsp65 (25 μg).

Immunofluorescent staining and flow cytometric analysis. Cells isolated from lymph nodes or spleen were incubated with antigen (MPB70 or Ag85B 0.2–5 μg/ml) for a week at 37 °C in 0.5% CO_2, harvested and stained for the lymphocyte markers interleukin-2R (IL-2R) (CD25) (LCTB2A, VMRD, Pullman, WA, USA), CD4 (SBUT-4, Centre for Animal Biotechnology (CAB), University of Melbourne, Australia), CD8 (SBU-T8, CAB) and γδ-T-cell receptor (TCR) (DU2-74, VMRD) [28]. Fluorochrome-conjugated secondary antibodies used were goat antimouse IgG2a/2b fluorescein isothiocyanate and goat antimouse IgG3 R-phycoerythrin (Southern Biotechnology Associates, Birmingham, AL, USA). Both primary and secondary antibodies were incubated with the cells at 4 °C for 30 min. After staining, the cells were fixed in fluorescence-activated cell sorter (FACS) lysis solution (Becton-Dickinson, Franklin Lakes, NJ, USA) and kept at 4 °C in the dark until analysis. Flow cytometric analyses were performed on a FACSCalibur flow cytometer (Becton-Dickinson).

Statistical analysis. The obtained optical density values in subclass-specific ELISA were tested for differences with respect to slope and intercept, according to the analysis of covariance described in Ref. [29]. Differences in cell populations found in FACS analysis were tested by one-tailed nonparametric statistical analysis employing the Mann–Whitney *U*-test using the INSTAT version 3.0 program (GraphPad, San Diego, CA, USA). Differences between proliferative, IFN-γ and skin-test responses of cattle were analysed employing unpaired one-tailed *t*-tests using INSTAT version 3.0. Differences were regarded as significant when the *P*-value was <0.05.

Results

Local anaesthetics in electroporation experiments did not cause trauma

Both cattle and goats were sedated before the vaccination procedure was carried out. Local anaesthesia was applied at the site of DNA injection with electroporation (see Table 1 for details). The animals were divided in two groups, the first group receiving plasmid DNA in combination with electroporation, whilst the other group only receiving plasmid DNA. The animals were observed throughout the experimental period. No immediate or long-term distress or discomfort, or any behavioural signs of muscle damage were observed during and after electroporation. In addition, no pathological changes were observed at the sites of injection and electroporation when muscle tissue samples taken postmortem were examined.

DNA vaccination of goats with MPB70 and Ag85B DNA induced humoral immune responses

MPB70- and Ag85B-specific serum IgG responses were determined pre- and postvaccination. No significant IgG levels were detected before vaccination. However, 3 weeks after vaccination, all electroporated goats showed significantly increased antigen-specific IgG responses against Ag85B ($P=0.032$) and MPB70 ($P=0.024$) when compared with the nonelectroporated group. Responding goats had average IgG titres of above 700 against Ag85B (Fig. 2A) and up to 400 against MPB70 (Fig. 2B). Over the next 8 weeks, IgG levels gradually declined in the responding animals in both groups and were no longer significantly different. After the boost injections at week 11, we observed a slight increase in the IgG titres in all animals, regardless of whether electroporation was performed or not. No changes in serum IgG titres were observed after the third boost.

Increased cellular immune responses after boost injections of DNA in goats

To assay cellular immune responses, whole blood samples were stimulated with the antigen MPB70, Ag85B or PPD-P. Proliferation and IFN-γ secretion assays showed no T-cell activity after the first vaccination. A slight increase was observed in proliferative activity after the first and second boost injections, although no differences between electroporated and control animals were observed (data not shown). Four weeks after the third boost injection, the animals were killed, and cells isolated from the lymph nodes and spleen were cultured together with the antigen for 1 week. No increase in the numbers of CD4-, CD8- or γδ-TCR-positive cells expressing the activation marker CD25 (IL-2R) was observed following culture. However, FACS analysis revealed a significantly increased population

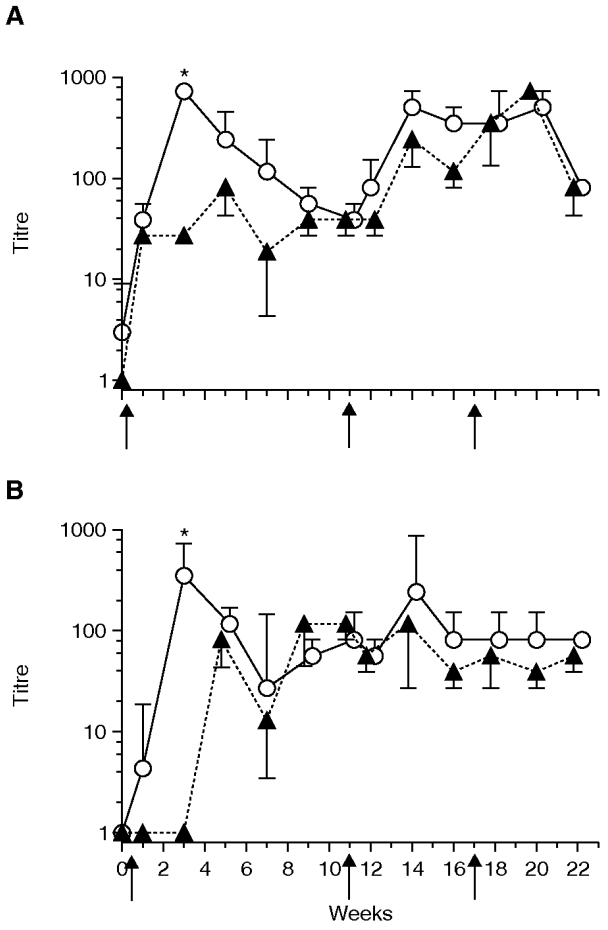

Figure 2 End point titres of antigen-specific serum immunoglobulin G (IgG) from the DNA-vaccinated goats. Panel A shows anti-Ag85B titre, whilst panel B shows anti-MPB70 titre. The end point titre was determined as the highest dilution of serum that would give a positive signal in the enzyme-linked immunosorbent assay (ELISA). The cut-off value was determined as the optical density value of the preimmune serum plus two times the standard deviation. The open symbols indicate the median response (+standard error of the mean (SEM)) of the electroporated animals ($n = 3$), while the closed symbols represent the median (–SEM) of the animals that did not receive electroporation ($n = 3$). The arrows on the abscissa indicate the initial (week 0) and the boost injections at weeks 11 and 17. Asterisk above data point at 3 weeks: $P < 0.05$.

($P < 0.05$) of large cells, with a low side-scatter profile in the cell samples from the right popliteal and subiliac lymph nodes draining the DNA-injected muscle (Fig. 3A,B) when compared with the corresponding left-side lymph node. This population was significantly larger ($P < 0.05$) in electroporated animals when compared with the nonelectroporated ones (Fig. 3C), and were CD4-positive (20–65% of gated cells), CD8-positive (5–24% of gated cells) or belonged to cell populations not defined by this staining procedure.

Immune responses induced after vaccination of cattle with pCMV4.65 encoding Hsp65

Three pairs of cattle twins that did not give rise to cellular (proliferation and IFN-γ responses) or humoral immune responses prior to vaccination (data not shown) were selected for the experiments and divided into two groups, with one sibling in each group (Table 1). The results in Fig. 4 depict the development of cellular immune responses after vaccination. No significant increases in Hsp65-specific cellular responses were observed in any of the animals 2 weeks after the primary vaccination.

At week 3 after the primary vaccination with pCMV4.65-expressing mycobacterial Hsp65, an increase in the number of IFN-γ-secreting cells was observed in all of the six vaccinated cattle (Fig. 4A). Increased proliferative responses (SI ≥ 3) were observed in five of the six animals (Fig. 4B). Significantly higher IFN-γ responder frequencies ($P = 0.03$) were observed in electroporated animals when compared with their nonelectroporated siblings (Fig. 4A). Similar findings, but not quite significant, were obtained when comparing the proliferative responses in electroporated and control animals, because PBMCs from two of the three electroporated cows showed higher responses when compared with their untreated siblings (Fig. 4B). The number of IFN-γ-secreting cells increased further following the booster vaccination given at week 3 in five of the six animals (tested at week 5, Fig. 4A). At week 5, the number of IFN-γ-secreting cells was more elevated in two of the electroporated animals when compared with their nonelectroporated siblings, although the relative differences were less pronounced between electroporated and nonelectroporated cattle at week 5 compared with week 3 of the experiment. At week 8 (5 weeks after the booster vaccination), no IFN-γ responses were demonstrated in any of the animals, regardless of whether electroporation was performed or not (Fig. 4A). In contrast, *in vitro* proliferative responses were observed in all the six animals at this time point. A higher response was observed in one electroporated animal only, compared with its nonelectroporated sibling (Fig. 4B). No antigen-specific serum IgG responses were observed in this experiment (data not shown).

Vaccination with Hsp65 DNA does not compromise the tuberculin skin test

To determine if *pCMV4.65* DNA vaccination encoding Hsp65 would compromise the specificity of the tuberculin skin test, we compared tuberculin skin tests at week 8 using avian (PPD-A) and bovine (PPD-B) tuberculin, as well as using recombinant Hsp65. A group of three animals experimentally infected with *M. bovis* served as the positive control. As shown in Fig. 5, all *M. bovis*-infected animals presented with pronounced skin reactions at the sites where PPD-B was injected, with considerably lower responses to PPD-A, correctly identifying them as *M. bovis*-infected. In contrast, none of the *hsp65* DNA-immunized animals, regardless of whether electroporation was performed or not, gave rise to positive skin reactions

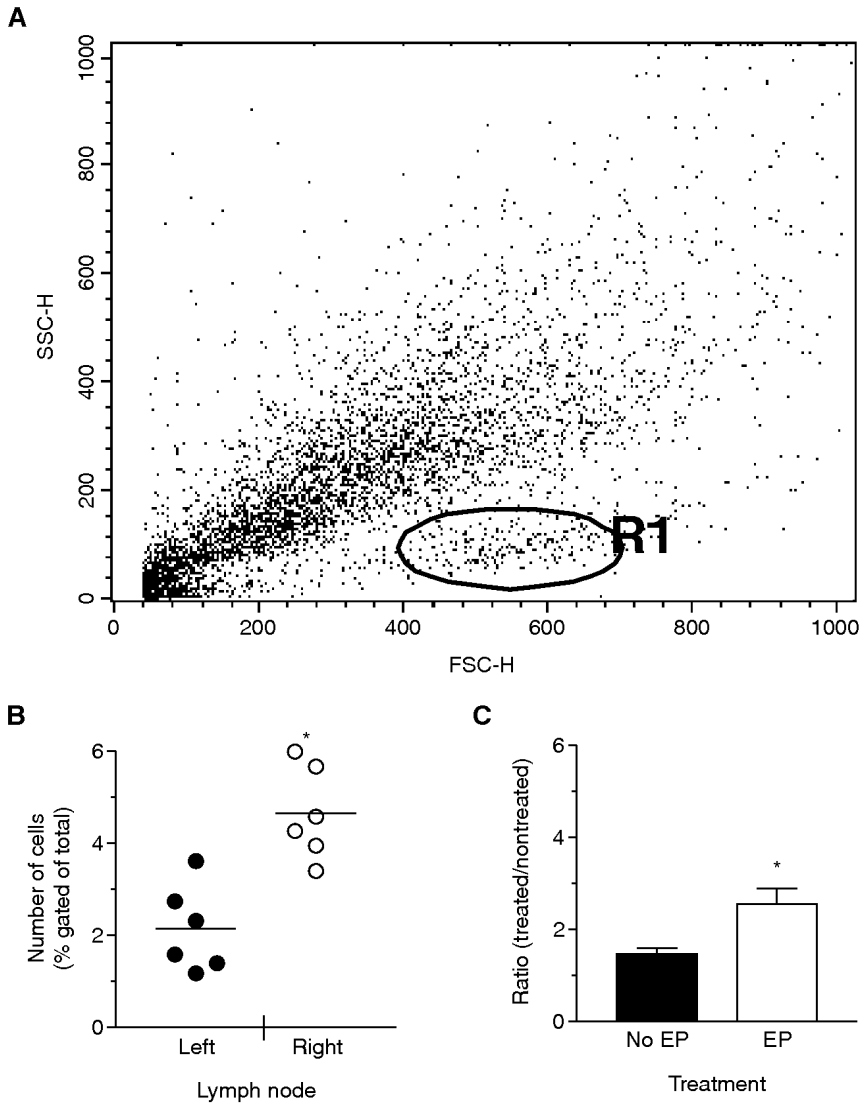

Figure 3 Fluorescence-activated cell sorter (FACS) analysis of cells harvested from the popliteus and subiliacus lymph nodes. Panel A shows the forward and side scatter of lymphoid cells from the right side. Panel B shows the number of large nongranular cells as per cent gated (R1) of total cells found in the lymph nodes harvested from the goats ($n = 6$). The lymph nodes on the right hand side (○) of the animal drain the DNA-injected muscle. Panel C shows the difference between the electroporated (EP) ($n = 3$) (□) and nonelectroporated (No EP) animals ($n = 3$) (■) in means of the ratio between the number of cells harvested and gated in R1 (panel A) from the lymph nodes on the right side (draining lymph nodes) and the number of cells in R1 from the corresponding lymph nodes on the left side. Asterisks indicate significant difference ($P < 0.05$).

after PPD-B ($P < 0.001$) injection, and they responded only weakly to PPD-A. Recombinant Hsp65 did not induce skin-test responses in DNA-vaccinated cows.

Cellular immune responses in the draining lymph nodes after protein antigen injection

Intrigued by the observation of strong proliferative responses in the absence of IFN-γ responses at week 8 (Fig. 4A,B), we set out to compare the number of IFN-γ-secreting cells in the peripheral blood and in the lymph nodes after 4 days of *in vivo* antigen stimulation. By performing skin test by injection of recombinant Hsp65 protein into the flanks of the cattle, we were able to determine if this protein boost would result in an increase in the number of IFN-γ-secreting cells in the draining lymph node when compared with the number of IFN-γ-secreting PBMCs. Figure 6 compares the results obtained in the peripheral blood 5 weeks post booster vaccination (week 8) with those obtained in the draining lymph nodes 4 days after the injection of Hsp65 protein. We detected proliferative responses in the peripheral blood, whilst the numbers of IFN-γ-secreting PBMCs were low, thus confirming the results shown in Fig. 4 for that time point before skin testing. In contrast, high numbers of IFN-γ-secreting cells were present in the draining superficial cervical lymph nodes (Fig. 6). No increase in the number of IFN-γ-secreting cells was observed in the lymph node cells prepared from the superficial cervical lymph nodes located on the other side of the animals' necks where no protein was injected (data not shown).

Discussion

DNA vaccination in combination with electroporation is an efficient way to increase the immune response in rodents [15, 16, 18, 19]. The present study describes the protocols to apply this method to farm animals, namely

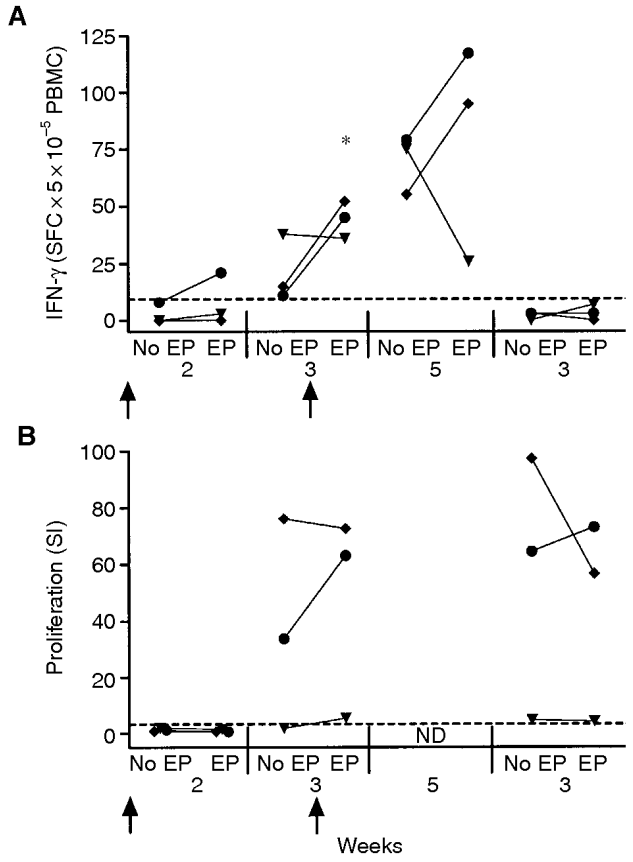

Figure 4 Cellular responses induced by pCMV4.65 vaccination of cattle. (A) Frequencies of IFN-γ-secreting cells (spot-forming cells, SFC) as measured by enzyme-linked immunospot (ELISPOT). Peripheral blood mononuclear cells (PBMCs) were stimulated *in vitro* with recombinant Hsp65 (5 μg/ml). Responses of SFC > 10 were deemed positive. (B) Proliferative responses of PBMCs stimulated *in vitro* with Hsp65. Results are expressed as stimulation indices (SIs) (cpm with Hsp65 divided by cpm of medium controls). Proliferative responses of SI ≥ 3 were deemed positive. Electroporated (EP) animals are indicated by open symbols and their corresponding nonelectroporated (No EP) siblings by closed symbols with the same shape. Dotted horizontal lines indicate cut-offs for positivity. The arrows on the abscissa indicate the initial (week 0) and the boost injection at week 3. Asterisk: significantly different between the No EP and EP group at the tested time point ($P < 0.05$).

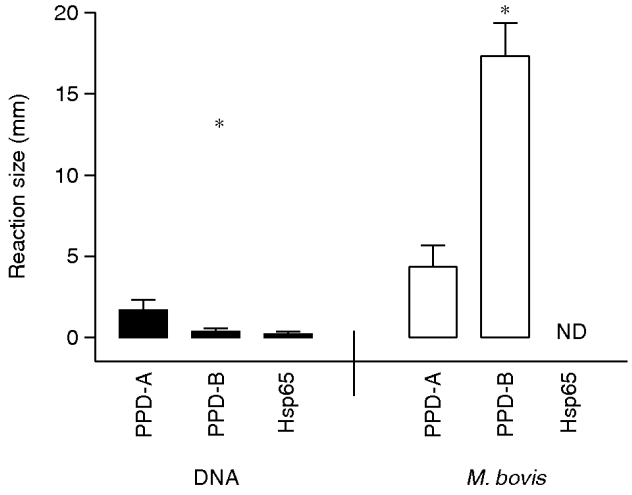

Figure 5 The specificity of the intradermal tuberculin test is not compromised in cattle by DNA vaccination with pCMV4.65. All DNA-vaccinated animals ($n = 6$) were tested using the intradermal comparative tuberculin test 5 weeks after the final immunization. Reactions to PPD-B, PPD-A and recombinant Hsp65 were determined 3 days after the tuberculin injection. A group of three animals, experimentally infected with *Mycobacterium bovis*, was included as the positive controls. Results are expressed as the mean reaction sizes in mm (+standard error of the mean (SEM)). ND, not determined. The asterisks indicate significant difference between the DNA-vaccinated and *M. bovis*-infected cattle tested with PPD-B ($P < 0.001$).

cattle and goats. As cattle and goats are frequently infected with *M. bovis*, we wanted to employ DNA vaccines that had been reported to protect against tuberculosis in rodents (Hsp65, MPB70 and Ag85B) [6, 7, 20] into larger animals.

Electroporation of small animals is normally performed under total anaesthesia [22, 30], but total anaesthesia of ruminants is not a preferred option, because it may result in ruminal disorder. The vaccination protocols described in this study demonstrated that local anaesthesia was sufficient to avoid trauma in the electroporated cattle and goats. Contractions of the muscle were observed during the procedure when local anaesthetics were used. However, these contractions were limited to the muscle volume

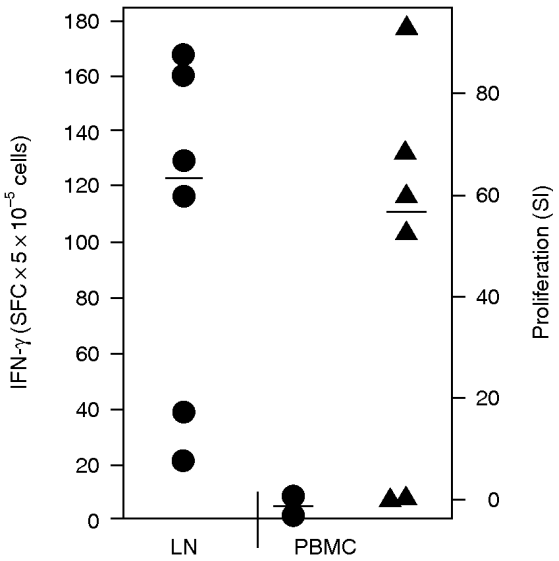

Figure 6 T-cell memory responses in draining lymph nodes after subcutaneous injection of Hsp65 protein. Frequencies of IFN-γ-secreting cells (spot-forming cells, SFC), indicated by circles, and proliferative responses, indicated by triangles, were determined in peripheral blood mononuclear cells (PBMCs) and lymph node (LN) cells prepared from the draining (superficial cervical lymph nodes) (left side) at week 8 of the experiment. Hsp65 protein was injected subcutaneously into the necks of the animals 4 days before blood sampling and postmortem examination to prepare lymph nodes. Cells were stimulated *in vitro* with recombinant Hsp65 (5 μg/ml). Horizontal lines indicate median values.

receiving electrical pulses, in contrast to more widespread reflex-induced contractions observed with full anaesthesia during the first immunization of the goats.

In small animals, DNA injection is followed by placement of electrodes on injected tissue and delivery of electrical pulses. Muscles in rodents are small, and the injected DNA solution volume is relatively large (50 μl). It is therefore simple to apply the electrical field at the injection site. In larger animals, however, it is technically demanding to apply a localized electrical field to the area of injection when the muscle is large and the volume is small. Hence, the electrodes described in this work, which deliver DNA and give electrical pulses at the same site, are advantageous for this purpose.

The data obtained indicate that goats injected with DNA in combination with electroporation had a strong primary humoral immune response against both Ag85B and MPB70 when compared with goats not electroporated, whereas the booster injections did not induce differences in antigen-specific antibody titres. The difference in IFN-γ and proliferative T-cell responses between electroporated and nonelectroporated cows was also more pronounced after the primary vaccination, although after the booster vaccination, the T-cell responses were still more elevated in two of the three *hsp65*-vaccinated cows that were electroporated, when compared with their nonelectroporated siblings. The notion that DNA vaccination followed by electroporation is efficient for the induction of primary immune responses is supported by both sets of results. Heterologous prime/boost strategies based on the induction (priming) of immunity by DNA vaccination followed by heterologous vaccine boosts with proteins [31], recombinant viruses like modified vaccinia virus Ankara (MVA) [32], or BCG [33] have been successfully applied to increase the protective efficacy of tuberculosis vaccines in rodents. Thus, delivery methods like *in vivo* electroporation, improving the efficiency of the DNA-priming step, would be highly desirable.

The observed difference in humoral and cellular immune responses between the species tested (goats and cattle) could be attributed to several factors. Firstly, different species and even different breeds of the same species can develop diverse immune responses after vaccination with the same antigens. For example, it has been reported that mice of H-2^b and $H2^d$ haplotype can develop Th1- and Th2-type responses, respectively, after BCG vaccination [34, 35]. Secondly, the balance between cellular and humoral immune responses induced following vaccination or infection could also be influenced by the nature of the antigens. MPB70 and Ag85B, for example, are secreted antigens, whereas Hsp65 is a nonsecreted stress protein. Interestingly, MPB70, together with its close homologue MPB83, is one of the main serological targets following *M. bovis* infection of cattle, badgers and deer [36–38]. Hsp65 is capable of inducing T-cell responses in infected cattle [39], but to our experience seldom induces a humoral immune response (Vordermeier *et al.*, unpublished observation).

The FACS analysis of lymphoid cells from lymph nodes draining the DNA-injected muscle in goats revealed a population of large cells with a low side-scatter profile (Fig. 3). This constitutes an interesting cell population that warrants further analysis. This cell population appeared to be composed of blast-like cells, although they were less granular than the 'conventional' blastoid cells we have previously observed in goats (Storset, data not shown). Unfortunately, the lack of relevant reagents detecting goat cell-surface markers limited further characterization of this cell population.

A key question for any vaccine is how long immunity will last after vaccination. The humoral immune response against Ag85B and MPB70 lasted for more than 20 weeks in all goats, in contrast to what has been shown in cows previously [5], with modest humoral response against MPB70 and none against Ag85A (75% homology with Ag85B [21]). In *hsp65* DNA-vaccinated cattle, we detected only low levels of IFN-γ-producing cells 5 weeks after the booster vaccination (week 8), whereas we were still able to demonstrate Hsp65-specific T-cell proliferation in PBMCs. This finding is in line with the current concepts of memory T-cell development in mice and humans [40–42]. It has been shown in these systems that the frequency of IFN-γ-producing *effector cells* decreased with the down-regulation of the immune response and that they were replaced by considerably lower frequencies of *effector memory* cells producing cytokines like IFN-γ, as well as by *central memory* cells producing mainly IL-2. These central memory cells are likely to be represented by the proliferating T-cell population that did not produce IFN-γ after the longer rest period. Supporting this interpretation is the observation that we were able to induce IFN-γ-producing effector cells within 4 days of subcutaneous injection of Hsp65 when determining the responder frequencies in the draining lymph nodes 6 weeks after the booster vaccination (Fig. 6).

The humoral responses in goats were obtained after one injection of 200 μg of DNA (100 μg of *mpb70* + 100 μg of *ag85b*). This is an improvement when compared with other studies with DNA vaccination of larger animals showing that multiple injections of DNA in the order of 1 mg are needed to obtain a detectable immune response [5, 26, 43]. A DNA vaccination study in humans showed that 2.5 mg of DNA was needed in order to obtain an immune response after one injection [44]. We have observed that 1 μg of DNA in combination with electroporation gives good immune responses in mice [19]. Previous studies suggest that 1 μg of DNA combined with electroporation generates about the same level of protein expression as 100 μg of DNA without electroporation [45]. Hence, the level of antigen expression is not necessarily

the explanation for improved response. Provided the amount of expressed protein exceeds a certain threshold level, the activation status and migratory properties of local antigen-presenting cells will be of greater significance for the outcome of the vaccination. Electroporation enhances entry of DNA into the cells, and it is essential to find the optimal DNA concentration as well as the optimal amount of DNA applied with this method. Dose–response studies will be required to allow an appreciation of the optimal procedure for the induction of sustained levels of immunity.

In conclusion, we have developed an applicable vaccination protocol for farm animals based on the injection of DNA, followed by electroporation that did not cause immediate or long-term trauma or damage to the vaccinated animals. In addition, DNA vaccination did not compromise the current skin test. Thus, this vaccination strategy could potentially be applied alongside conventional tuberculin skin-test-based control strategies, which is in agreement with the results of others [5] (Vordermeier, unpublished data). DNA vaccination with electroporation is an efficient method for the induction of strong primary immune responses; and as such, it may become an important part of heterologous prime boost strategies.

Acknowledgments

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